ISSN: 0974-5343
IJMST (2017), 7(3):18-24

Comparison of Local Cytokine Expression in Vaginal Secretions of Women with Normal and Abnormal Microbiota

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ABSTRACT:
Cytokines play important roles in the mediation of immune responses locally or systemically in every individual. Knowledge of the local expression of cytokines when there is a deviation from normal to abnormal microbiota in the urogenital tract gives an insight to the extent of the innate immune responses to abnormal microbiota. Bacterial vaginosis (BV) is the most common example of changes in urogenital flora. The concentrations of IL-2, IL-10 and TNF-α in vaginal secretions of women with abnormal microbiota was determined by ELISA techniques. Statistical analysis show significant differences ($P < 0.05$) in the concentrations of the cytokines of the different groups of the women. There were significantly elevated levels ($p < 0.05$) of TNF-α and IL-10 in women with BV, when compared those of women with intermediate flora. The results show a predominant local pro-inflammatory response to deviations from normal to abnormal microbiota in the urogenital tract.
Keywords: TNF-α, IL-2, IL-10, Abnormal microbiota, Bacterial vaginosis.

INTRODUCTION

The vagina is the first line of physical and immunological defence against invading flora in the urogenital tract [1, 2]. Potentially pathogenic microorganisms, including protozoans, yeast, bacteria, and viruses, initiate the immune response and result in increased vaginal secretion of immune stimulating molecules [1, 3].

Bacterial vaginosis (BV) is an inflammatory and sometimes recurring syndrome of the lower genital tract, caused by a deviation from normal microbiota of the urogentigal tract of women. It is associated with proliferation of a number of organisms such as *Gardnerella vaginalis*, *Mobiluncus* species, *Mycoplasma hominis* and *Peptostreptococcus* sp associated with loss of vaginal *Lactobacilli* [4, 5]. Bacterial vaginosis has been reported as a risk factor for pelvic inflammatory diseases and cervicitis that can lead to pre-term birth, premature rupture of membranes and chrioamniotis in pregnant women [6-8].

The physiological defence mechanisms against foreign microorganisms are mediated by the innate and adaptive immune responses [7]. The effector phases of both innate and adaptive immunity are often regulated by peptide molecules known as cytokines [2]. The cytokine cascade is composed of a series of activities that rapidly generate innate and adaptive immune responses which often determine the outcome and the clinical course of infection [8].

We chose to examine the cytokines IL-2 and TNF-*α*, because it is representative of a type 1 T-helper lymphocyte (Th1)-mediated response that regulates cytotoxic T lymphocytes which in association with phagocytic cells and soluble antimicrobial factors are responsible for clearing infections in the genital tract. Additionally, the cytokine IL-10 was chosen because it is a regulatory cytokine representative of the humoral immune response. This study was carried out to assess the concentrations of the aforementioned cytokines in vaginal secretions of women with bacterial vaginosis.

MATERIALS AND METHOD

Study Population

The study population consisted of women presenting with abnormal vaginal discharge at the clinical consult of the medical microbiology and parasitology department of the University of Port Harcourt Teaching Hospital, Rivers state, Nigeria within a six month period (January – June 2015). Eighty (80) Female patients presenting with abnormal vaginal discharge were consecutively recruited for the study. Ethical approval was obtained from the ethics committee of the hospital and verbal consent was sought from the women before they were recruited for the study.

ISSN: 0974-5343
IJMST (2017), 7(3):18-24

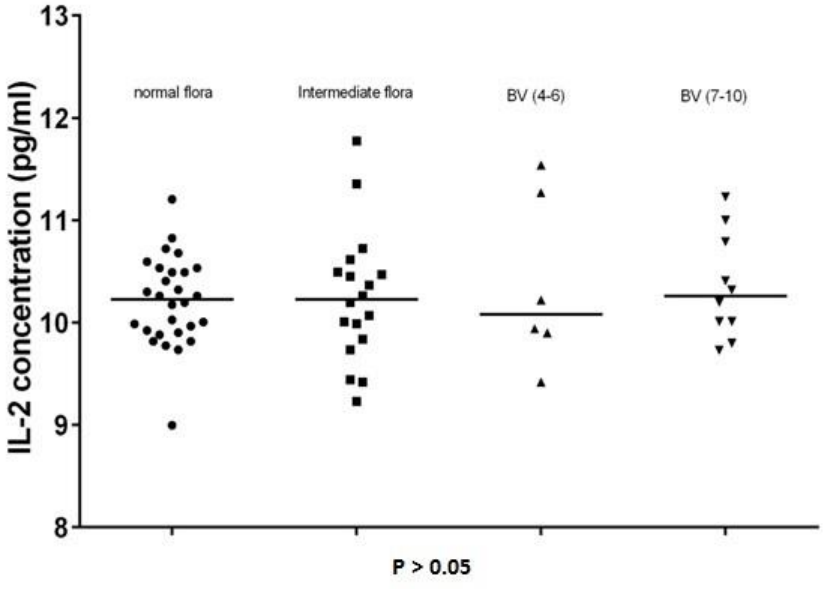

Fig 1. Column scatter plot of IL-2concentrations in vaginal secretions of subjects with normal microbiota (Nugent score of 0 – 3; n = 29), intermediate flora (Nugent score of 4-6, no clue cells, n = 17), bacterial vaginosis (Nugent score of 4 – 10, with clue cells; n =24). Solid lines indicate median values for each group. Cytokine levels were compared across groups by use of Kruskal-Wallis test. There was no significant difference ($p > 0.05$) in cytokine levels of the different groups when compared to cytokine levels in normal microbiota

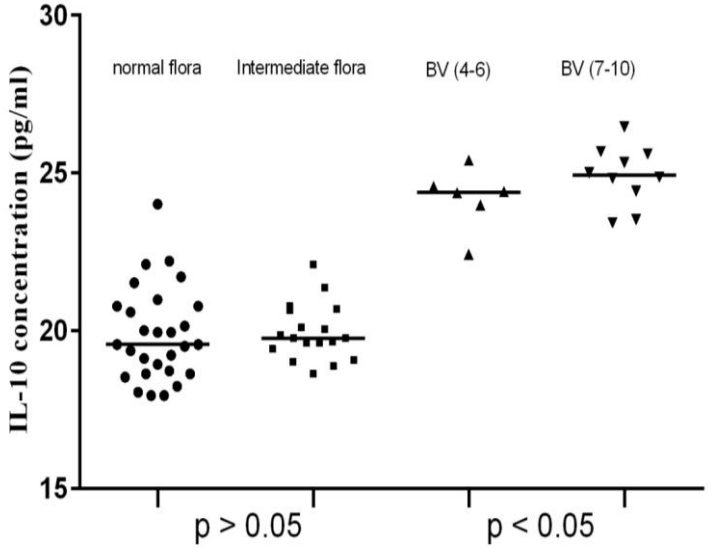

Fig 2. Column scatter plot of IL-10 concentrations in vaginal secretion samples from subjects with normal microbiota, intermediate flora and bacterial vaginosis (BV). Solid lines indicate median values for each group. Cytokine levels were compared across groups by use of Kruskal-Wallis test. If significant differences were noted, Dunn's multiple comparison test was used to calculate individual p values between the groups. There was no significant difference ($p > 0.05$) between cytokine levels of subjects with normal microbiota and intermediate flora, with a significant difference ($p < 0.05$) observed in cytokine levels of subjects with BV compared to normal microbiota.

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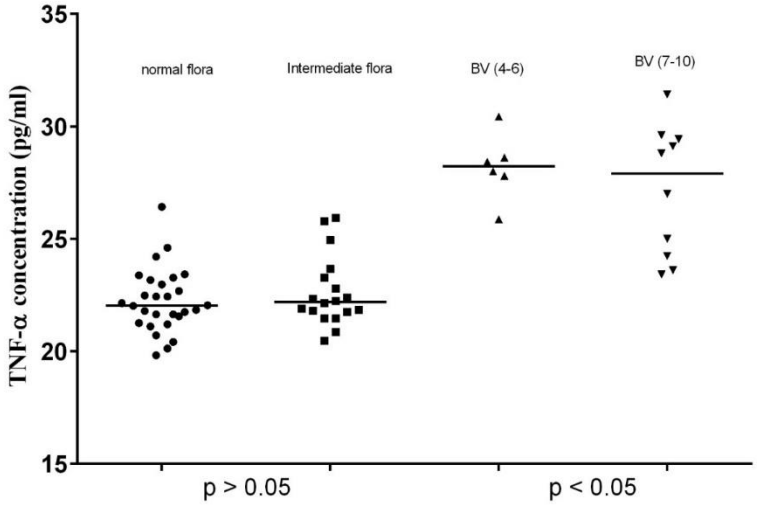

Fig 3. Column scatter plot of TNF-α concentrations in vaginal secretion samples from subjects with normal microbiota, intermediate flora and bacterial vaginosis (BV). Solid lines indicate median values for each group. Cytokine levels were compared across groups by use of Kruskal-Wallis test. There was no significant difference ($p > 0.05$) between cytokine levels of subjects with normal microbiota and intermediate flora, with a significant difference ($p < 0.05$) observed in cytokine levels of subjects with BV compared to normal microbiota

Specimen Collection

At the point of High Vaginal Swab (HVS) collection for standard care, two HVS were collected using Dacron coated swabs. One of the swabs was used for microscopic analysis and the other was placed in 3mL of phosphate buffered saline (PBS) and centrifuged for 10 minutes. The supernatant was aspirated and stored in aliquots at -20^{o}C for subsequent analysis by Enzyme Linked Immunosorbent Assay (ELISA) as described by Stute et al [9].

Microscopic analysis of specimen

Thin smears of each swab sample was made on grease-free glass slides as described by Jorgensen et al [10]. The smears were Gram-stained viewed under the x100 objective of the microscope for the presence of bacterial morphotypes as described [11]. Gram stained smears were evaluated for BV by microscopic evaluation of the relative numbers of *Lactobacillus* morphotypes, *Gardnerella* or *Prevotella* morphotypes, and gram-negative curved rods such as *Mobiluncus* species present in vaginal smears and Nugent scores were given according to the presence of bacterial morphotypes seen and presence of clue cells as described by Nugent et al [12]. The samples were separated into three groups based on their Nugent score as follows.

Group A – Normal microbiota (Nugent score of 0 – 3)

Group B – Intermediate Flora (Nugent score of 4 – 6, with no clue cells)

Group C – Bacterial vaginosis (Nugent score of 4 – 10, with clue cells)

Cytokine evaluation

TNF-α, IL-2 and IL-10 were quantified by use of ELISA kits (Avivabio Systems, CA, USA), following the manufacturer's instructions. The average of the duplicate readings for each known standard was used to generate a standard curve and cytokine concentrations of the test samples

were determined with corresponding optical densities on the standard curves.

Statistical analysis

Kruskal-Wallis and Dunn's post's tests was used to compare cytokine levels across multiple groups. All statistical tests were performed at a 5% significance ($p < 0.05$) using the Prism software package (Graphpad Software Version 6.0).

RESULTS

The figures 1, 2 and 3 are column scatter plots of the IL-2, IL-10 and TNF-α concentrations respectively. There were no significant differences ($p > 0.05$) in IL-2 concentrations across the different groups of the subjects. However, there were significant differences ($p < 0.05$) in IL-10 and TNF-α concentrations of women with BV when compared to cytokine concentrations of women with normal microbiota and intermediate flora respectively.

DISCUSSION

The concentrations of IL-2, IL-10 and TNF-α, were statistically similar ($p > 0.05$) among women with normal microbiota and those with intermediate flora. This may be an indication of a minimal immune response, probably due to the early stage of colonization by these pathogens and the absence of clue cells which indicate a successful colonization of the epithelial cells by abnormal microbiota as reported in related studies [13, 14].

Cytokine concentrations observed in women with abnormal microbiota were in contrast to the findings of similar studies by Anton et al [7] and Hedges et al [13] which reported mean IL-10 concentration of 9.8pg/ml and a mean TNF-α concentration of 10.2 pg/ml, with no significant differences between IL-10 and TNF-α concentrations. The mean IL-2, IL-10 and TNF-α concentrations recorded in this study were slightly higher than those previously reported [7, 13].

This study shows significantly elevated levels of TNF-α and IL-10 in women with abnormal microflora, when compared to women with normal microflora and intermediate flora. Also, local expression of TNF-α was significantly higher than IL-10 and IL-2 in the different groups of women. This may be an indication of a dominant local pro-inflammatory immune response to the presence of abnormal microbiota that may be potentially pathogenic as reported in previous studies [14, 15]. The TNF-α mediates inflammatory responses at the vagina by the up-regulation of leukocyte adhesion molecules on the endothelial cells and serves as a chemoattractanct for neutrophils, neutralizing the invading pathogens [8, 14].

The cytokine IL-10 enhances B-cell proliferation and antibody production for the neutralization and elimination of the pathogens [8, 13]. Its relatively lower expression in comparison to TNF-α in women with abnormal microflora maybe an indication of a relatively low localized humoral mediated immune response to the presence of abnormal microflora such as *Gardnerella vaginalis*, *Mobiluncus* species, *Mycoplasma hominis* and *Peptostreptococcus* sp.

The expression of IL-2 induces the development of Th1 cells, which induces macrophage activation and delayed type hypersensitivity [14-16]. IL-2 may also exhibit pleitropic functions in mounting immune responses by dampening them when they tend to be exaggerated [16, 17]. There was no significant difference in IL-2 concentration between women with normal and abnormal microbiota. This suggests that there is no hypersensitivity-type reaction to abnormal microbiota in the urogenital tract of the women with abnormal microbiota.

CONCLUSION

Varying concentrations of cytokines in the vaginal secretions of women with normal microbiota, intermediate flora and bacterial vaginosis were observed in the study. These cytokines aid in the maintenance of the dynamic balance between resident microbial flora, physical and chemical constituents of the vaginal ecosystem. The pattern of cytokine expression suggests a predominant localized pro-inflammatory response to abnormal microflora in women with abnormal microbiota.

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ISSN: 0974-5343
IJMST (2017), 7(3):18-24

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ETHICAL APPROVAL

PROFINFLAMATORY CYTOKINES ASSOCIATED WITH VAGINITIS

We refer to your letter dated 4th June, 2014 requesting for Ethical Approval of your research project titled "**Profinflamatory Cytokines Associated with Vaginitis**".

After a critical appraisal of your proposal by the University of Port Harcourt Teaching Hospital Ethical Committee and the Research Ethics Group of the Centre for Medical Research and Training, College of Health Sciences, University of Port Harcourt, approval is hereby given to you to commence your study.

Note the following:

1. The study can only be started after it is approved by the examining body.

The Hospital reserves the right to withdraw this approval if at any time during the conduct of the study you infringe on the ethical regulations of the Hospital or the ethical rights of your study subject.

B. J. Thom-Manuel (Mrs.)
Secretary
for: Chairman

ISSN: 0974-5343
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INFORMED CONSENT FORM

This is to certify that I ..... willingly give my permission for the collection of High Vaginal Swab samples for the purposes of this study alone. All details of the study have been explained to me and I understand there is no risk to me in the course of this study.

....
Patient Thumb Print/ Signature **Date**

....
Researcher **Date**